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Therapeutic potential of ndv-2k17 strain in canine mammary gland tumor

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Abstract
Canine mammary tumor is the most common tumor in adult female dogs. Surgical intervention is the oldest and most widely accepted treatment option, even though it has limitations in presence of micro-metastasis and lymphatic invasion. Oncolytic viral therapy offers a new dimension in cancer treatment by eliminating malignancies by direct targeting and lysis of the cancer cells, leaving the noncancerous tissue unharmed. The present study was carried out to evaluate the therapeutic effect of Newcastle disease virus as an adjunct to surgical excision of canine mammary tumor. Six bitches under group-I having grade-III mammary tumor were subjected for four intravenous doses of purified Newcastle disease virus post operatively whereas, other six bitches under group-II also with grade-III mammary tumor were treated by surgical treatment alone. The six bitches of group-1, received the viral therapy, showed better result in terms of overall survivability and disease free survivability than that of the group-II bitches. Surgical treatment alone was not found to be sufficient in canine mammary tumors with lymph node metastasis. Hence, viral mediated therapy with Newcastle disease virus can be used as an adjunct to surgical excision in canine mammary tumor cases with metastatic lesion in the regional lymph nodes.

Keyword: canine, mammary tumor, viral therapy, micrometastasis.

Introduction
In last two decades neoplasm related death in pets increased drastically, may be due to delayed diagnosis and lack of availability of effective treatment options for complete cure of malignant tumor. Mammary gland tumor is the commonest of all the tumors in adult female dogs with 50 per cent malignancy (Brodey *et al.*, 1983) [1]. The status of the regional lymph nodes (RLN) plays an important role in determining the invasiveness and taking the decision on the treatment protocol to be followed, as RLN being the commonest site of early metastasis in canine mammary gland tumor (Misdorp and Hart, 1979) [3, 4]. Among the available treatment protocols (viz. surgery, chemotherapy, radiotherapy, diathermy and immunotherapy), surgical intervention is the oldest and most widely accepted treatment option for canine mammary gland tumors. But surgical treatment alone is not sufficient in presence of micro-metastasis and lymphatic invasion (Gilbertson *et al.*, 1983) [2].

Oncolytic viral therapy is one of the newly emerging therapeutic alternatives for cancer, offering a new dimension in cancer treatment by eliminating malignancies by direct targeting and lysis of the cancer cells, leaving the noncancerous tissue unharmed. The combination of the viral therapy with the conventional surgical treatment as an adjunct augments the efficacy of the treatment by selective lysis of the micrometastases (Ockert *et al.* 1996) [5].

The oncolytic property of Newcastle disease virus was demonstrated by Cassel and Garrett (1965) [9], Heicappell *et al.* (1986) [8], Pecora *et al.* (2002) [6], Krishnamoorthy *et al.* (2006) [7] and Leonidas *et al.* (2007) [11] in human cancer patient. The antineoplastic efficacy of Newcastle disease virus is associated with three properties of the virus, viz., selective replication in the tumor cells, direct cytotoxicity against cancer cells, and immuno-stimulatory capacity of the virus (Fournier *et al.*, 2012) [10]. So far such study has not been carried out involving clinical cases of tumors in animals. Here we studied the therapeutic potential of Newcastle disease virus as an adjunct to surgical excision in clinical cases of canine mammary gland tumor.

Patient Enrollment

Twelve bitches (irrespective of breeds) with advanced mammary gland tumors, showing evidence of metastasis in the regional lymph nodes in fine needle aspiration cytology were enrolled for the study. Entry criteria included at least one tumor of more than 5 cm in diameter, no radiographic evidence of metastasis in the internal organs, more than 6 years of age and a life expectancy of at least 2 months. Hematology results minimum entry requirements included 7.5g/dL hemoglobin, 3,000 WBCs/µL, 1,500 neutrophils/µL and 1, 00,000 platelates/µL. Also the serum biochemistry result requirements included creatinine and BUN levels less than 2 times the upper limit of normal and transaminase level less than 2.5 times the upper limit of normal. Patients documented to have heart problems, active viral infection or uncontrolled bacterial infection and history of being in contact with poultry were excluded.

All the twelve enrolled bitches were randomly divided into two equal groups Viz. Group 1 and Group 2. The group 1 bitches were treated with postoperative intravenous viral therapy as an adjunct to surgical excision of mammary tumor, whereas the group 2 bitches were treated with conventional surgical excision only.

Table 1: Clinical examination of mammary tumor in Group 1 and Group 2 animals

Group	Animal No.	Size (cm)	Location of the tumor					Ulceration	LN involvement
			CrT	CdT	CrA	CdA	Ing		
Gr 1	1	6.2	-	-	-	Right	Right	-	Ing
	2	7.4	-	Left	Left	Left	-	+	Axi, Ing
	3	5.8	-	-	-	Right	-	+	Ing
	4	9.4	Right	Right	-	-	-	+	Axi
	5	7.9	-	-	Left	Left	Left	-	Ing
	6	10.2	-	-	-	-	Right	-	Ing
Mean ± S.E.		7.81±1.73							
Gr 2	1	5.6	-	-	-	Right	-	-	Ing
	2	15.7	-	-	Left	Left	Left	-	Ing
	3	10.7	-	-	-	Left	Left	+	Ing
	4	9.4	Right	Right	-	-	-	+	Axi
	5	8.6	-	-	-	Right	Right	+	Ing
	6	8.4	-	-	-	Left	Left	+	Ing
Mean ± S.E.		9.73±3.37							

CrT – cranial thoracic, CdT – caudal thoracic, CrA – cranial abdominal, CdA – caudal abdominal, Ing – inguinal, Axi – axillary, LN – lymph node.

The Virus

The Newcastle disease virus strain NDV2K17/ Quail/ Chennai/ India/1998 classified as genotype-2, based on the phylogenetic analysis of the fusion protein cleavage site, was used for the study. The viral strain was kindly provided by the Department of Animal Biotechnology, Madras Veterinary College, Chennai, under the ICAR- Niche area of Excellence programme. The virus was isolated from a quail and was pathotyped as velogenic based on the dibasic amino acids in the fusion protein cleavage (^{112}**RRQKRF**^{117}).

Cells Used

A continuous cell line, the MDCK (Madin Darby Canine Kidney), kindly supplied by School of Animal Biotechnology, GADVASU, Ludhiana, was used for passaging and propagation of the NDV.

Passage of Ndv-2k17 Strain in Mdck Cell Line

The MDCK cells were sub-cultured in Dulbecco Modified Eagle Medium (DMEM) with low glucose, L-Glutamine and 110 mg/L Sodium pyruvate (Hyclone®) to get a confluent growth of monolayer within 48 hours of incubation. Then the cell culture medium in the culture flask was discarded and 0.5 ml of the viral suspension was spread over the cell monolayer and incubated at 37˚C for one hour with 5% CO_2 for adsorption of the virus on to the cells. After one hour the culture flask was added with medium and incubated at 37˚C with 5% CO_2 for 72 to 96 hrs and was examined under an inverted microscope.

Viral Infectivity Test By Reverse Transcription Polymerase Chain Reaction (Rt-Pcr)

The infected cells were followed for the development of cytopathic effect during the course of the infection and also tested for the presence of the viral genome. The viral nucleic acid RNA was extracted from the infected cell culture fluid using Roche High pure nucleic acid kit (Roche, USA) and the cDNA was synthesized using High capacity cDNA synthesis kit (Invitrogen, CA). Polymerase chain reaction (RT-PCR) was performed targeting the region encompassing the 3' end of Matrix and 5' end of fusion protein gene (genomic position 4331 to 5090) using the following degenerate primer pair (Tirumurugaan *et al.*, 2011) [12].

FP 5' GAGGTTACCTCYACYAAGCTRGAGA 3'

RP 5' TCATTAACAAAYTGCTGCATCTTCCCWAC 3'

Purification

The pooled cell culture fluid was freeze thawed and clarified by centrifugation at 8000rpm for 20 minutes at 4˚C. The supernatant was subjected to crude pelleting at 32,000 rpm for 3 hrs in an ultracentrifuge. The pellet was resuspended in TNE buffer (50mM Tris, 100mM NaCl, 1mM EDTA, pH 7.4) and overlaid on an equal volume of discontinuous gradient consisting of 30 per cent and 60 per cent sucrose. The tubes were centrifuged at 24,000 rpm for 2 hour at 4˚C which yielded the virus band between 40 to 50 per cent sucrose gradient, which was collected, diluted in TNE buffer and finally centrifuged at 30,000 rpm for 2 hour at 4˚C to pellet the virus. The purified virus pellet was aliquoted and stored at -80 ˚C for further use (Xiangpeng *et al.*, 2012) [13]

Plaque Assay

The purified viruses were serially diluted in growth medium from 10^{-1} to 10^{-6} dilutions, added on to MDCK cells in 6 well culture dishes and were overlaid with methyl cellulose. The cells were followed over the next 96 hrs under inverted microscope for the presence of cytopathic effect. The titre of

the purified virus suspension was calculated which was expressed as viral plaque forming unit (PFU) expressed in per ml basis.

Cytology

The invasiveness of the tumors was diagnosed with the help of fine needle aspiration cytology of the tumor mass, regional lymph nodes and nipple aspiration cytology. All the 12 cases were diagnosed as grade III mammary gland tumor in a four stage TNM classification system as per the WHO, based on the tumor size, lymph node involvement and distant metastasis, as reported by.

Surgical Treatment

Regional mastectomy keeping 2cm three dimensional safety margin were performed to remove the tumor mass along with the RLN in all the twelve cases. A course of antibiotic and analgesic were given to all the cases postoperatively and the skin sutures were removed on 10^{th} postoperative day. The surgical wound healed without any complication in 10 bitches, whereas in two bitches there were seroma formation and wound dehiscence. Then the six bitches of Group 1, were subjected to postoperative viral therapy. All the excised tumor mass and regional lymph nodes were sent for histopathological study.

Regimen for the Viral Therapy

We calculated the body surface area of the patients in meter square (M^2) using the formula suggested by Rosenthal (1981) [15]. The six bitches of Group 1, were administered with four doses of purified Newcastle disease virus through intravenous route on 7^{th}, 28^{th}, 48^{th} and 68^{th} postoperative day, single dose containing 5.09 x 10^4 PFU per square meter body surface area.

All the six animals developed fever (104 ± 0.5˚F) and anorexia following first injection which lasted for 3 to 5 days. Mild conjunctivitis was noticed in two animals which subsided following supportive therapy. Reductions in body weight were also noticed in all the six bitches of group 1, during the period of viral therapy which came to normal after the end of the therapy.

Overall Survivability (Os) and Disease Free Survivability (Dfs)

All the animals were monitored for recurrence or distant metastasis of the primary tumor by routine general clinical examination, haematobiochemical examination, thoracic radiography and abdominal ultrasonography for a period of one year following treatment. The overall survivability is the time period from the day of surgery to the day of death due to the same cause. The disease free survivability is the time period from the day of the surgery to the day of first detection of recurrence of primary tumor or distant metastasis in any other organs due to the excised primary tumor (Santos *et al.*, 2013) [14]. The six dogs of group-2, received the viral therapy, lived for the entire study period without any difficulty whereas, the average overall survivability and disease free survivability of group-2 animals were recorded to be 283 days and 221 days respectively. There were distinct radiographic evidences of distant metastasis of the primary tumor in four bitches of group-2, out of which three died during the study period due to the metastatic lesions in the lungs and other organs as confirmed by autopsy.

Table 2: Outcome of treatment in relation to histopathological classification of the tumors

	Ani. No.	FNAC	Histopathology of mammary tumors	Metastasis in RLN	OS (days)	DFS (days)
Gr 1	1	Carcinoma	Mixed mammary tumors	Present	365	365
	2	Carcinoma	Squamous cell carcinoma	Present	365	365
	3	Carcinoma	Mixed mammary tumors	Present	365	365
	4	Carcinoma	Papillary adenocarcinoma	Present	365	365
	5	Carcinoma	Tubular adenocarcinoma	Present	365	365
	6	Carcinoma	Mixed mammary tumors	Present	365	365
Mean ± S.E.					**365**	**365**
Gr 2	1	Carcinoma	Tubular adenocarcinoma	Present	254	180
	2	Carcinoma	Mixed mammary tumors	Present	68	45
	3	Carcinoma	Mixed mammary tumors	Present	365	213
	4	Carcinoma	Papillary adenocarcinoma	Present	279	156
	5	Carcinoma	Papillary adenocarcinoma	Present	365	365
	6	Carcinoma	Tubular adenocarcinoma	Present	365	365
Mean ± S.E.					**283**	**221**

The specificity and sensitivity of the fine needle aspiration cytology should be correlated with the histological findings of malignancy which could be used in clinical staging of the disease and was helpful in deciding the treatment protocol (Daniela *et al.*, 2009) [19].

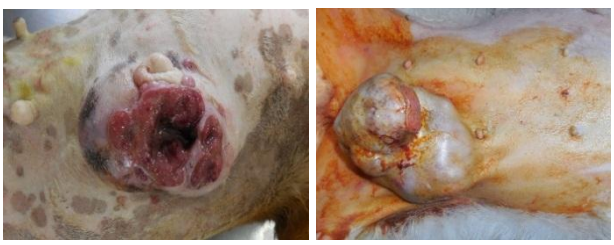

Fig 1: Gross mammary tumor

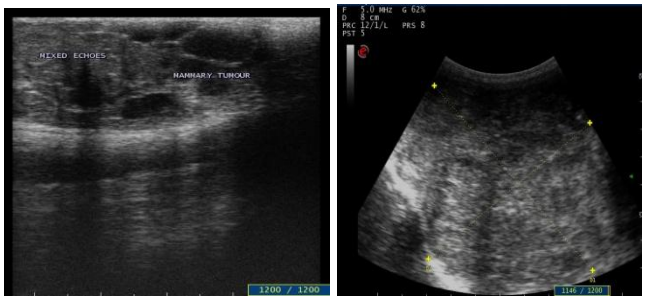

Fig 2: Ultrasono graphic pic of the tumor mass showing heterogenous and homogenous echogenicity

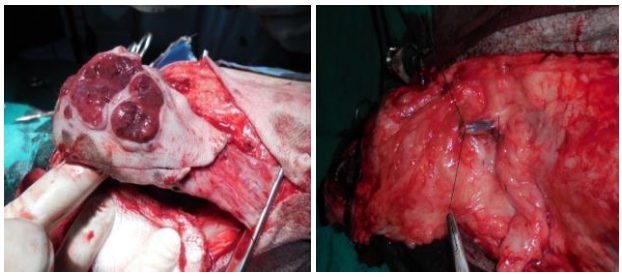

Fig 3: Intraoperative image of mammary tumor

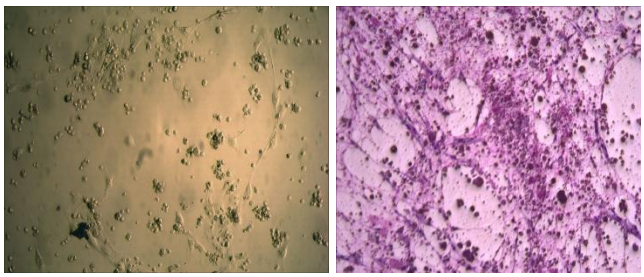

Fig 4: Propagation of NDV 2K17 strain in MDCK cell line

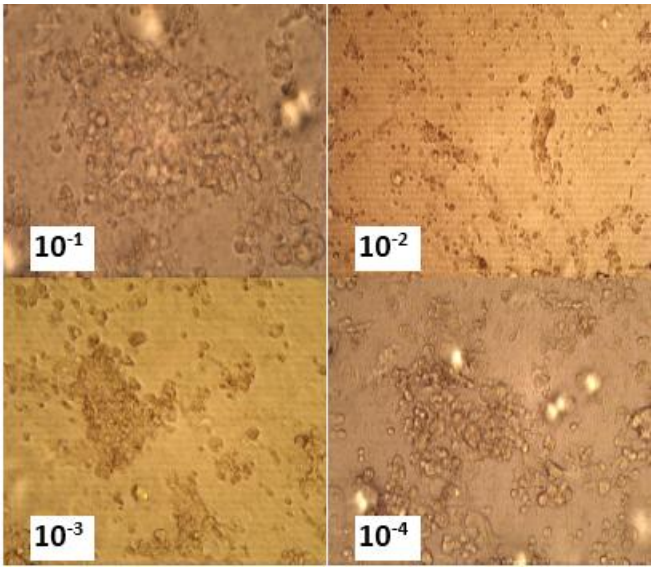

Fig 5: Plaque assay of NDV 2K17

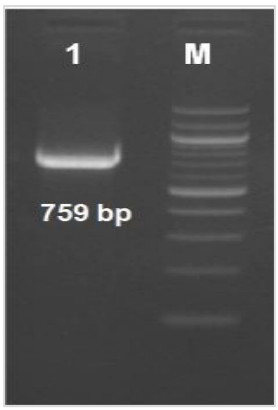

Fig 6: Reverse Transcription PCR of the 3' end of Matrix and 5' end of fusion protein gene (genomic position 4331 to 5090) from the RNA extracted from infected MDCK cells

Discussion

Oncolytic virotherapy represents a promising option in cancer treatment due to the selective nature of the viruses for infection and replication. Cancer cells had altered microenvironment which is exploited by the oncolytic viruses for targeting as stated by Ries and Brandts (2004) [17] and Singh *et al.* (2012) [18]. This study revealed that in canine mammary tumors, viral therapy as an adjunct to surgical excision, prolonged the survivability of the patient which was in accordance with the findings of Heicappell *et al.* (1986) [8], who reported disappearance of the micrometastasis and prolongation of life in all animals following viral therapy using NDV compared to the control group. This might be due to the selective nature of the Newcastle disease virus to propagate and cause death of the neoplastic cells keeping the other healthy cells unharmed (Pecora *et al.*, 2002 and Krishnamoorthy *et al.* 2006) [6, 7]. We stand with the views of Patil *et al.*, (2012) [20], that the combination of oncolytic viruses with the available routine conventional treatment options may be a key to the optimization of the oncolytic viral therapy in dogs with neoplasms.

Histopathological study of the regional lymph nodes revealed presence of metastatic lesions in all the 12 cases. Chang *et al.* (2005) [23], also performed histopathological examination of the surgically excised regional lymph nodes and reported that regional lymph node metastasis was most likely to be seen in dogs with mammary tumors more than 5 cm in diameter. According to Tuhoy *et al.* (2009) [22], Szczubial and Lopuszynski (2011) [21] and Santos *et al.* (2013) [14], regional lymph nodes were the most common site of early regional metastasis in canine mammary tumors and were associated with an increased risk for the development of recurrence or distant metastasis and tumor associated death.

All the 6 animals in group-1, treated with intravenous injection of Newcastle disease virus showed mild fever, mild conjunctivitis, slight reduction in body weight and anorexia for 3-4 days following viral injection. Freeman *et al.* (2006) [24], Leonidas *et al.* (2007) [11] and Lam *et al.* (2011) [25] reported mild conjunctivitis, laryngitis, hypotension, and flu-like symptoms including mild fever, headache, tiredness and weakness as general side effects of oncolytic Newcastle disease virus therapy in humans. Pecora *et al.* (2002) [6], opined that accumulative toxicity was not associated with repeated Newcastle disease virus strain administration.

Use of oncolytic viral therapy with Newcastle disease virus as an adjunct to surgical excision in canine mammary tumor cases with metastatic lesion in the regional lymph nodes improved the survivability when compared to surgical intervention alone. However, further studies are required to delineate the mechanistic evidence, dosage regimen and screening for shedding of the virus in canine patients with adjunct oncolytic viral therapy.

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